Dear Editor,

Thank you for providing us with the editorial and peer review comments on our manuscript entitled “Identification of coding and noncoding RNA-binding proteins by using click chemistry-assisted RNA-interactome capture (CARIC)”. We have added more details to the critical steps and reorganized the highlighted steps according to the comments. Several steps have been combined or reorganized in order to meet the page limit. The revisions are summarized in the following line-by-line responses.

**Editorial comments:**

*“1.3.1: What is the concentration of EDTA-free protease inhibitor cocktail?”*

**Response**: The EDTA-free protease inhibitor cocktail is purchased in the form of tablets. We added one tablets for 50 mL Pre-Lysis buffer. We’ve added this note to the Table of Materials.

*“1.3.5: Mention needle gauge.”*

(The step number 1.3.5 has been changed to 1.3.4)

**Response**: We thank the editor for pointing this out. The needle gauge has been added.

*“1.3.6,2.1.1: Mention rotation speed.”*

(The step number 1.3.6 has been changed to 1.3.5)

**Response**: We thank the editor for pointing this out. The rotation speed has been noted in these two steps.

*“1.4.2,1.4.4,: Do you centrifuge? If so, mention speed in g and duration.”*

**Response**: Yes, we centrifuge the tubes. We’ve added more details, including rotation speed and duration time.

*“2.1.2: Specify the duration on the magnet.”*

**Response**: The duration on the magnet is specified.

*“2.3.1: Methanol %?”*

(The step number 2.3.1 has been changed to 2.4.1)

**Response**: The methanol concentration is added in the text.

*“3.3.1,3.3.4,3.3.5,3.4.5,: Mention centrifuge speed and duration.”*

(The step number 3.3.4, 3.3.5, and 3.4.5 have been changed to 3.3.2, 3.3.3, and 3.4.3, respectively)

**Response**: We thank the editor for pointing this out. The centrifuge speed and duration have been added in these steps.

*“3.3.4: Mention rotation speed.”*

(The step number 3.3.4 has been changed to 3.3.3)

**Response**: Step 3.3.3 is a repetitive step of 3.3.2. The rotation speed is added in step 3.3.2.

*“4.1.2: Mention gel %.”*

**Response**: The acrylamide percentage of the gel is added.

*“4.2.2: For filming, please describe the steps (in brief is okay). What is the silver concentration /percentage?”*

**Response**: We thank the editor for this advice. We have added a brief workflow of the silver staining step.

*“6.1.1, 6.2.2: Please rewrite in the imperative voice.”*

(We believe “6.1.1” is a typo for “6.2.1” because step 6.1.1 was already written in imperative voice.)

**Response**: We thank the editor for pointing this out. These two steps have been rewritten in imperative voice.

*“The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.”*

**Response**: We thank the editor for the kind advice. We have reorganized the highlighting protocols so that all sub-steps of the highlighted steps are included in the highlighting.

*“Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.”*

**Response**: We have combined some short or repetitive steps to shorten the manuscript length. The combined and reorganized steps are (old step number to new step number): 1.3.3 and 1.3.4 to 1.3.3; 1.3.6 and 1.3.7 to 1.3.5; 1.4.4 and 1.4.5 to 1.4.4; 2.2.2 and 2.2.3 to 2.2.2; 2.2.4 and 2.2.6 to 2.2.3; 2.2.5 to 2.3.1 and 2.3.2; 2.3.7 and 2.3.8 to 2.4.7; 3.3.3 and 3.3.4 to 3.3.3; 3.4.3, 3.4.4, and 3.4.5 to 3.4.3; 3.5.1 and 3.5.2 to 3.5.1.

*“The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.”*

**Response**: We thank the editor for the kind advice. We have carefully reorganized the highlighted steps in a logical way.

*“Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.”*

**Response**: The length of highlighted steps has been re-calculated in the right way.

*“Notes cannot be filmed and should be excluded from highlighting.”*

**Response**: The notes have been excluded from highlighting.

*“Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.”*

**Response**: We thank the editor for the reminder. We didn’t include any software steps in highlighting.

*“Please ensure that the manuscript title best reflects the filmable content (i.e. the portions you highlight).”*

**Response**: We thank the editor for the reminder. We have checked the filmable content.

*“JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.”*

**Response**: We have added paragraphs in the Discussion section regarding the demanding contents, including the detail description of a critical step, the limitation of our technique, and the future applications.

*“JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are StageTips, MaxQuant,”*

**Response**: We thank the editor for pointing this out. However, StageTips and MaxQuant are not commercial names. The StageTip (stop-and-go-extraction tip) is a technology developed by Professor Mann and colleagues for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics (see reference 30 of the manuscript). The MaxQuant is a software developed by Professor Mann and colleagues for proteomic data analysis and quantification (see reference 31 of the manuscript).

*“Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as software used, cell lines, etc.”*

**Response**: The information of the cell line and software is included in the table of materials.

**Peer-review comments:**

**Reviewer #1**

*“1.3.6. Incubate the lysate at 4 °C with gentle rotation for 1 hr.  
\* Is the abovementioned step necessary for homogenization?”*

**Response**: We thank the reviewer for pointing this out. This step allows the complete denaturing of proteins, which is critical for minimizing the background signal from uncross-linked RBPs or non-RNA binders.

*“1.4.2. Concentrate each fraction by using a 15-mL ultrafiltration tube 177 (molecular weight cutoff of 10 kDa) till the volume is smaller than 1 mL.  
\* Further clarification is needed for the following step, how long and what speed is needed for spinning the ultrafiltration columns.”*

**Response**: More details of this step including centrifuge speed and duration have been added to the protocol.

*“2.2.4. Incubate the reaction mixture for 2 hr at room temperature with vortex.  
\* Agitation? Rotation? Or occasional vortexing?”*

(The step number 2.2.4 has been changed to 2.2.3)

**Response**: We thank the reviewer for pointing this out. We incubate the reaction mixture on an orbital shaker at 800 rpm. This description has been added to the protocol.

*“Line 215 volume of lysate. After incubation of 2 hr at room temperature  
\* After incubating 2hrs at room temperature.”*

**Response**: We thank the reviewer for pointing this out. The description of this step has been reorganized.

*“3.4. Elution of the captured RNP.  
\* RNP ◊ RBPs  
\* How efficient is the protocol for eluting biotinylated molecules directly from Streptavidin beads if compared to eluting RBPs off the beads by degrading RNA using RNase?  
\* Why is RNase treatment done after elution, rather than as part of the elution?”*

**Response**: In this step, biotinylated RNAs are eluted along with the cross-linked binding proteins. Thus we believe “RNP” is a more appropriate description than “RBP”. We tried RNase A digestion for elution instead of using biotin elution buffer. However, it didn’t work well even with very high concentration (~1 mg/mL) of RNase A. It is possible that the RNase is blocked by streptavidin and the cross-linked RBPs. The biotin elution buffer contains high concentration of detergent which will denature the RNase. Therefore, we need to remove most of the detergent by ultrafiltration after elution to avoid inactivation of RNase.

*“4.1.3) … If there are significant biotin signals remains…  
\* Improve the grammar!”*

**Response**: We have corrected it in the revised manuscript.

*“5.1.8) Dehydrate the gel pieces in neat acetonitrile (ACN) and rehydrate with 200 μl of 10 mM dithiothreitol (dissolved in 50 mM ABC) at 56 °C for 45 min.  
\* This step needs to be clarified. How much ACN should be added? How many times?  
\* Likewise, step 5.1.10”*

**Response**: We use 1 mL neat ACN for gel pieces of each lane. For most cases, two rounds of dehydration are needed. We’ve added this note to the protocol.

*“5.3.1.5) Wash the tip with 50 μl of 80% ACN in 10 mM ABC (pH 10.0).  
\* How the pH of ABC should be adjusted to 10 mM? By adding ammonium hydroxide to ammonium formate? It needs to be clarified.”*

**Response**: We thank the reviewer for pointing this out. The pH of ABC is adjusted by adding ammonium hydroxide. We’ve added this note to the protocol.

*“6.1.1) Reconstitute the dried peptide fractions from step 5.3.7 in 15 μl water containing 0.1% formic acid  
\* The acidity of the samples should be check on a pH strip.”*

**Response**: We thank the reviewer for pointing this out. We’ve added this note to the protocol.

*“6.1. Peptide analysis by LC-MS/MS.  
\* The MS method needs to be described in detail here.”*

**Response**: Detailed description of MS method has been added to the protocol.

*“6.3.1) Perform a moderated t-test implemented in limma to test the Log2-fold change against zero from at least three biological replicates.  
\* What does zero stand for? The negative control? If so, explain.  
\* Describe in detail how to use the limma package.”*

**Response**: The data used in the moderated t-test analysis is the Log2-fold change value of the protein abundance in the experimental sample compared to that in the control sample. If one protein’s Log2-fold change is zero, the abundance of this protein is equal in the experimental sample and the control sample, indicating this protein is not enriched at all.

The detailed description of how to use the limma package for moderated t-test has been added to the protocol.

**Reviewer #2**

*“In Figure 1A, the difference between samples with or without RNaseA treatment is not so obvious.”*

**Response**: The main signal of cross-linked RNPs are located at high molecular weight region (>250 kDa) in the form of smeared bands as described in the figure legend. This signal is completely abolished by RNase digestion. The sharp bands at lower molecular weight region are non-specific background signals.

*“In line 88, it's better to add a reference for '3~5% of the eukaryotic transcriptome'.”*

**Response**: We thank the reviewer for pointing this out. A reference has been added for this statement.

*“In line 89, change 'on' to 'interacting with'.”*

**Response**: We thank the reviewer for pointing this out. We have corrected this in current manuscript.

*“In line 254, it's better to keep consistence between '10 mL' and '10-mL' in line 260.”*

**Response**: We thank the reviewer for the kind advice. Now the description is consistent.

*“How much time does it take to concentrate the lysate? This is not mentioned in the protocol. And, could this affect the integrity of RNA?”*

**Response**: The centrifuge speed and duration have been added to the protocol. This step normally takes 2-3 hr. The RNAs will be partially degraded after this step. However, as we mentioned in the discussion section, the ultrafiltration step is critical for this method and partial degradation of RNAs will not significantly affect the results.

*“THPTA should be spelled out on first use.”*

**Response**: We thank the reviewer for pointing this out. The full name of THPTA is now presented on first use.

We thank the editor and reviewers again for their comments. We hope that you find the paper is now acceptable for publication. Of course, we would be happy to address any additional concerns, or make any other amendments that you deem necessary.

Thank you for considering our revised paper.

Sincerely,

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